



Year: 2009

TRAF2 must bind to cellular inhibitors of apoptosis for tumor necrosis factor (tnf) to efficiently activate nf-kappab and to prevent tnf-induced apoptosis

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Abstract: Tumor necrosis factor (TNF) receptor-associated factor-2 (TRAF2) binds to cIAP1 and cIAP2 (cIAP1/2) and recruits them to the cytoplasmic domain of several members of the TNF receptor (TNFR) superfamily, including the TNF-TNFR1 ligand-receptor complex. Here, we define a cIAP1/2-interacting motif (CIM) within the TRAF-N domain of TRAF2, and we use TRAF2 CIM mutants to determine the role of TRAF2 and cIAP1/2 individually, and the TRAF2-cIAP1/2 interaction, in TNFR1-dependent signaling. We show that both the TRAF2 RING domain and the TRAF2 CIM are required to regulate NF-kappaB-inducing kinase stability and suppress constitutive noncanonical NF-kappaB activation. Conversely, following TNFR1 stimulation, cells bearing a CIM-mutated TRAF2 showed reduced canonical NF-kappaB activation and TNF-induced RIPK1 ubiquitylation. Remarkably, the RING domain of TRAF2 was dispensable for these functions. However, like the TRAF2 CIM, the RING domain of TRAF2 was required for protection against TNF-induced apoptosis. These results show that TRAF2 has anti-apoptotic signaling roles in addition to promoting NF-kappaB signaling and that efficient activation of NF-kappaB by TNFR1 requires the recruitment of cIAP1/2 by TRAF2.

DOI: <https://doi.org/10.1074/jbc.M109.072256>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-58109>

Journal Article

Accepted Version

Originally published at:

Vince, J E; Pantaki, D; Feltham, R; Mace, P D; Cordier, S M; Schmukle, A C; Davidson, A J; Callus, B A; Wong, W Wei-Lynn; Gentle, I E; Carter, H; Lee, E F; Walczak, H; Day, C L; Vaux, D L; Silke, J (2009). TRAF2 must bind to cellular inhibitors of apoptosis for tumor necrosis factor (tnf) to efficiently activate nf-kappab and to prevent tnf-induced apoptosis. *Journal of Biological Chemistry*, 284(51):35906-35915. DOI: <https://doi.org/10.1074/jbc.M109.072256>

TRAF2 MUST BIND TO cIAPs FOR TNF TO EFFICIENTLY ACTIVATE NF- κ B AND TO PREVENT TNF-INDUCED APOPTOSIS

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Running title: Function of the TRAF2-cIAP1/2 interacting motif

TNF receptor associated factor-2 (TRAF2) binds to cIAP1 and cIAP2 (cIAP1/2) and recruits them to the cytoplasmic domain of several members of the TNF receptor superfamily (TNFRSF), including the TNF-TNFR1 ligand-receptor complex. Here we define a cIAP1/2 interacting motif (CIM) within the TRAF-N domain of TRAF2, and use TRAF2 CIM mutants to determine the role of TRAF2 and cIAP1/2 individually, and the TRAF2-cIAP1/2 interaction, in TNFR1 dependent signaling. We show that both the TRAF2 RING domain and the TRAF2 CIM are required to regulate NF- κ B inducing kinase (NIK) stability and suppress constitutive non-canonical NF- κ B activation. Conversely, following TNFR1 stimulation, cells bearing a CIM mutated TRAF2 showed reduced canonical NF- κ B activation and TNF induced RIPK1 ubiquitylation. Remarkably, the RING domain of TRAF2 was dispensable for these functions. However, like the TRAF2 CIM, the RING domain of TRAF2 was required for protection against TNF induced apoptosis. These results show that TRAF2 has anti-apoptotic signaling roles in addition to promoting NF- κ B signaling and that efficient activation of NF- κ B by TNFR1 requires the recruitment of cIAP1/2 by TRAF2.

The inhibitor of apoptosis (IAP) family is comprised of baculoviral IAP repeat (BIR) containing proteins, of which several also bear a RING domain that is capable of acting as a ubiquitin E3 ligase (1). cIAP1 and cIAP2 are two RING containing IAPs whose amplification or genetic mutation has been associated with cancers, and may promote tumor cell survival (2-8). These highly conserved IAPs were initially identified as components of a TRAF2 containing complex bound to the cytoplasmic domain of TNFR2 (9), and have subsequently been implicated in the regulation of signaling by several more receptors of the TNF superfamily (TNFSF) (10-21). While the BIR1 domain of cIAP1/2 mediates binding to TRAF2 (17,22,23), the complementary binding region of TRAF2 is unknown, and the relative roles of TRAF2 and cIAP1/2 E3 ligase activity in TNF superfamily (TNFSF) signaling remain unclear.

Genetic deletion of TRAF2 in the mouse results in early post-natal lethality that is caused by increased NF- κ B-mediated TNF production and increased cellular sensitivity to TNF killing (12,24,25). Most TRAF family members, including TRAF2, bear RING E3 ubiquitin ligase domains that are believed to catalyze K63-linked ubiquitylation (26,27). One of the proposed targets of TRAF2 RING E3 ligase activity is RIPK1 (28,29), which is modified with K63-linked polyubiquitin chains upon TNFR1 activation. This modified TNFR1

complexed RIPK1 has been proposed to serve as a platform for the recruitment and/or activation of TAB2/TAB3/TAK1 and NEMO/IKK α /IKK β kinase complexes (30-32), which target the NF- κ B inhibitor I κ B for K48-linked polyubiquitin-mediated proteasomal degradation, to initiate canonical p65-dependent NF- κ B gene transcription.

As in the case of TRAF2, genetic deletion of cIAP1, or IAP antagonist induced loss of cIAP1/2, also sensitizes cells to TNF killing (16,19,33-36). Also like TRAF2, both cIAP1 and cIAP2 have been reported to ubiquitylate RIPK1 (10,15,16). Consistent with the idea that cIAP1/2 play an important role in the activation of NF- κ B by TNF, the removal of cIAP1/2 limits TNF from inducing NF- κ B by the canonical pathway (10,15,16). While it remains possible that both TRAF2 and cIAP1/2 play similar roles in modulating TNFR1 signaling through RIPK1 ubiquitylation, it is unknown whether cIAP1/2 act indirectly by activating the E3 ligase activity of TRAF2, or if cIAP1/2 act in combination with TRAF2 to directly ubiquitylate RIPK1, or alternatively, whether cIAP1/2 are the major E3 ligases for RIPK1 and TRAF2's role is to recruit them to TNFR1.

Both TRAF2 or cIAP1/2 deletion causes constitutive non-canonical NF- κ B activity, which occurs by stabilization of the NF- κ B inducing kinase NIK, IKK α activation, and processing of the non-canonical NF- κ B subunit p100, to the active p52 form. Activation of non-canonical NF- κ B can occur by proteasomal or lysosomal degradation of cIAP1, TRAF2 or TRAF3 and is mediated by ligand induced activation of TNFSF receptors such as TNFR2, CD40, BAFFR or FN14 (12,13,17,20,21,37-44). On the basis of these recent studies (11,12,33) it appears that TRAF3 binding to NIK recruits the TRAF2-cIAP1/2 module and that cIAP1/2 mediate K48-linked ubiquitylation of NIK, which reduces NIK to undetectable levels in resting cells and prevents spontaneous non-canonical NF- κ B signals.

In this study we have identified TRAF2's cIAP1/2 interaction motif (CIM), and used TRAF2 CIM mutants defective in cIAP1/2 binding to determine how cIAP1/2 and TRAF2 coordinately suppress non-canonical NF- κ B activation and regulate signaling from TNFR1.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfections, Constructs and Lentiviral Infections. All cell lines were maintained at 37°C, 10% CO₂ in DMEM supplemented with 8% FBS and passaged twice weekly. Transient transfections using 1 μ g DNA per 10cm tissue culture plate were performed with Effectene™ (Qiagen) using manufacturer's protocols. The NF- κ B lentiviral reporter vector pTRH1 mCMV NF- κ B dscGFP was purchased from System Biosciences. Cre-recombinase and SV40 Large T antigen were cloned into the lentiviral vector pFU. Mouse TRAF2 and the CIM and RING domain TRAF2 mutants were cloned into pEF-NFLAG and the 4-hydroxy tamoxifen inducible lentiviral vector pF 5x UAS, which we have described in detail elsewhere (19,45). Full length cIAP1 and the BIR1 constructs were cloned into pEF-NHA. Complete sequence of all constructs can be obtained upon request. Lentiviral particles were generated as previously described (17,19).

Antibodies. The primary antibodies used were anti-FLAG (Sigma: F-3165), anti- β -actin (Sigma: A-1978), anti-RIPK1 (Mouse, BD Transduction Laboratories: 610458. Human, Pharmingen: 551042), anti-p65 (Santa Cruz: SC-372), anti-phospho-Ser p65 (Cell Signalling: #3033), anti-phospho-Ser32/36 I κ B α (Cell Signaling #9246), anti-I κ B α (Cell Signaling: #9242), anti-TNFR1 (Abcam: #19139), anti-TRAF2 (Pharmingen: 558890, Santa Cruz: SC-876, Calbiochem: AP1040), anti-cIAP1 (Alexis, ALX-803-335), anti-cIAP pan (RnD: MAB3400), anti-cIAP2 (in house, 16E6-3), anti-TRADD (Santa Cruz: SC-7868), anti-NIK (Cell Signaling: 4994), anti-p100/p52 (Cell Signaling: 4882).

Generation of MEFs. Knock-out MEFs were generated from E15 embryos using standard procedures and infected with SV40 Large T antigen expressing lentivirus. TRAF2 conditional knock-out MEFs were similarly generated from TRAF2 LoxP/LoxP E15 embryos. To delete TRAF2 the transformed MEFs were infected with a cre expressing lentivirus (pFU cre SV40 Hygro) and deletion was confirmed by PCR and Western blotting.

Death Assays. Cells were seeded on 12 well tissue culture plates at approximately 70 percent confluency and allowed to adhere. Compound A (500 nM), described in (19), or

human Fc-TNF α (60 ng/ml) were added to cells for 24 hrs and cell death measured by Propidium Iodide (PI) staining and flow cytometry. In each sample 10,000 events were measured, and the cell death (% PI positive cells) quantified.

Western Blotting and Immunoprecipitations. Immunoprecipitations were performed as previously described (17,19,46) with some minor alterations. Briefly, 4×10^7 MEFs were treated in the presence or absence of human FLAG-TNF at 1 μ g/ml for 5 minutes. Cells were lysed in IP-lysis buffer (30 mM Tris-HCl, pH 7.4, 120 mM NaCl, 2 mM EDTA, 2 mM KCl, 1 % TX100, Roche complete protease-inhibitor cocktail) at 4 °C for 30 minutes. The lysates were centrifuged at 15,000g for 30 minutes. 0.5 μ g of FLAG-TNF was added to the non-stimulated (time=0) control and the TNF-R1 complex was precipitated using M2 beads (Sigma) for 16 hours. The beads were washed five times with IP-lysis buffer and eluted with 2x LDS buffer (NuPAGE, Invitrogen). Proteins were separated by SDS-PAGE (NuPAGE, Invitrogen) and analyzed by Western blotting. Membranes were stripped with 50 mM Glycine (pH 2.3) and reprobed with other antibodies.

Immunofluorescence. MEFs grown overnight on glass coverslips were treated with Fc-TNF (60ng/ml) for the indicated times, immediately fixed with 3.2% paraformaldehyde for 20 minutes, washed in PBS, and permeabilized with 0.5% TritonX-100 for 5 minutes. Cells were blocked, incubated with p65 antibody, washed four times with PBS, and then incubated with anti-rabbit Alexafluor488 conjugated secondary antibody (Invitrogen) and washed four times again. All blocking steps and antibody incubations were performed with PBS containing 1% BSA for 30 minutes. Cells were viewed on a Olympus BX50 fluorescence microscope and SPOT RT imaging software using a x40 objective.

RESULTS

Identification of a cIAP1 and cIAP2 interacting motif (CIM) in TRAF2. The cIAP1 BIR1 domain and several key residues within this region have been shown to mediate cIAP1 binding to TRAF2 (17,22,23). However, the region of TRAF2 required for cIAP1 and cIAP2 binding has not been clearly defined since yeast two hybrid experiments suggested the TRAF-N

domain was important (9). Our own yeast two hybrid analysis suggested that a TRAF2-N domain containing fragment, residues 262-349, is sufficient for cIAP1/2 binding (Fig. 1A, 1C and not shown). To verify the ability of this region to bind cIAP1 *in vivo*, we performed co-immunoprecipitation experiments in 293T cells. Immunoprecipitation of full length FLAG-TRAF2 confirmed that it interacts with the cIAP1 BIR1 domain and can also bind two BIR1 domain truncation mutants lacking the first 22 and 39 residues (Fig. 1B) (17,22,23). Furthermore, a FLAG-TRAF2-N (aa262-349) construct was sufficient by itself to immunoprecipitate HA tagged full length cIAP1 or the cIAP1 BIR1 domain alone (Fig. 1C). Experiments using purified cIAP1 BIR1 and TRAF2-N domains demonstrated that the binding between these two regions was direct (Fig. 2D).

While the crystal structure of truncated TRAF2 is available, only the C-terminal part of the TRAF-N domain is present (47,48) making it difficult to identify the potential interaction interface between TRAF2-N and cIAP1 BIR1. We therefore examined the conservation of the TRAF2-N domain from a number of organisms to predict which residues might be important for the TRAF2-cIAP1 interaction (Fig. 1D). TRAF1 has also been shown to interact with cIAP1/2 (9,49) and was also therefore included in the alignment. This analysis revealed a cluster of residues spanning amino acids 283 to 294 that were highly conserved across all TRAF2 species examined and also TRAF1 (Fig. 1D). Importantly, this region showed little conservation with TRAF3 or *Drosophila* TRAF2 (Fig. S1A and S1B), none of which bind cIAP proteins. We therefore created several alanine point substitutions between or near mouse TRAF2 residues 283-294, a TRAF2 deletion spanning the residues 283-293, and a TRAF2 truncation by introducing a stop codon at residue D310 (Fig. 1D).

TRAF2 residues 283-293 are required for binding to cIAP1 and cIAP2. To determine which residues of TRAF2 are important for binding cIAP1 and cIAP2 we transiently transfected the indicated FLAG-TRAF2 constructs (Fig. 1D and 2A) into 293T cells and examined their binding to endogenous cIAP1 and over expressed cIAP2 by FLAG immunoprecipitation and Western blot. As before TRAF2-N (262-349) and wildtype (WT)

TRAF2 bound cIAP1 and, as expected, TRAF1 also immunoprecipitated cIAP1. The TRAF2 deletion, $\Delta 283-293$ did not bind detectable amounts of cIAP1 or cIAP2, and the double alanine point substitution EVE292AVA mutant showed drastically reduced binding to both cIAP1 and cIAP2 (Fig. 2A-2D). Single alanine point substitution of either E292 or E294 was less disruptive of cIAP1 and cIAP2 binding than mutation of both residues in the EVE292AVA mutant (Fig. 2B). TRAF2 E283A (and EN283AA) showed slightly reduced cIAP1 binding, but the other TRAF2 mutants, including the TRAF2 D310 truncation mutant and a Δ RING mutant lacking the first 87 amino acids, all interacted with cIAP1 like wild type TRAF2 (Fig. 2A, 2B). Similar decreases in TRAF2 mutant binding to cIAP1 were also observed in other cell types, such as D645 glioma cells (Fig. S1C). These mutations are unlikely to affect TRAF2 trimerization because the cIAP1/2 interacting motif was not present in the trimeric TRAF2 crystal structure (47,48). Consistent with the idea that the TRAF2 mutations do not affect TRAF2 structure dramatically, the TRAF2 mutants with reduced binding to cIAP1/2 interacted with endogenous TRADD and RIPK1 to the same extent as the wild type protein (Fig. 2C) (50). Experiments using purified cIAP1 or cIAP2 BIR1 domain proteins with purified wild type, EVE292AVA or $\Delta 283-293$ TRAF2-N domain proteins confirmed that BIR1 binding to TRAF2-N was direct and that mutant TRAF2-N binding was reduced (EVE292AVA) or abolished ($\Delta 283-293$) (Fig. 2D). The cIAP1/2 interacting motif (CIM) of TRAF2 therefore spans residues 283-294, and mediates the direct interaction between the TRAF-N domain of TRAF2 and the BIR1 domain of cIAP1.

A functional TRAF2 CIM and RING domain are required to inhibit non-canonical NF- κ B. In unstimulated WT cells TRAF3 binding to NIK recruits a TRAF2-cIAP1/2 complex to allow cIAP1/2 mediated NIK degradation and inhibition of spontaneous non-canonical NF- κ B (11,12,33,51). Deletion of TRAF2 or cIAP1 therefore results in constitutive non-canonical NF- κ B signaling (Fig. 3A, 3C)(17,19,52). Complementation of TRAF2 knockout MEFs with the EVE292AVA TRAF2 mutant, that is still able to bind low amounts of cIAP1/2 (Fig. 2B, 2D), was able to partially restore NF- κ B activity in TRAF2 knockout cells

to the basal levels observed in WT cells (Fig. 3B, 3C), while the TRAF2 $\Delta 283-293$ CIM mutant completely failed to do so, as measured by increased NIK levels, p100 processing to p52 and NF- κ B GFP reporter activity (Fig. 3B, 3C). The TRAF2 RING domain mutant also failed to reduce TRAF2 knockout NIK levels and p100 processing (Fig. 3C) despite the fact that it binds cIAP1 and cIAP2 (Fig. 2B). This shows that cIAP1/2 mediated K48-linked ubiquitylation and degradation of NIK relies on TRAF2 as an adaptor protein, and that the TRAF2 RING domain is also essential for the ability of cIAP1/2 to degrade NIK.

The TRAF2 CIM is required for resistance to TNF induced cell death. TRAF2 knockout MEFs immortalized with the SV40 large T antigen are highly susceptible to cell death induced by TNF treatment alone (17) (Fig. 4A). These TRAF2 knockout cells showed no significant difference in their ability to be killed by TRAIL and only a slight, potentially insignificant, difference in their ability to be killed by FasL (Fig. 4A), suggesting TRAF2 primarily provides protection against TNF induced apoptosis. Similarly, SV40 large T immortalized wild type (WT) MEFs that have been treated with the IAP antagonist Compound A to remove cIAP1, cIAP2 are killed by TNF treatment within 24 hrs (19) (Fig. 4A). Because cIAP1/2 are recruited by TRAF2, these data indicate that either cIAP1/2 or TRAF2, or both, confer protection against TNF killing but do not distinguish between these possibilities.

Reintroduction of inducible WT TRAF2, or the E283A TRAF2 mutant that is able to bind cIAP1/2, into TRAF2 knockout MEFs almost fully restored protection against TNF killing (Fig. 4B, 4C). However, induction of the TRAF2 CIM $\Delta 283-293$, EVE292AVA or RING domain mutants in TRAF2 knockout MEFs failed to prevent TNF induced cell death (Fig. 4C). These data demonstrate that cIAP1/2 binding to TRAF2 is crucial to prevent apoptosis induced by TNF and suggests that any interaction cIAP1/2 may have with RIPK1 (14) is not sufficient for TNF resistance in the absence of their binding TRAF2. Significantly, these results indicate that the RING domain of TRAF2 is also required for TNF resistance (Fig. 4C), even though this construct retains the ability to bind both cIAP1 and cIAP2 (Fig. 1B). Therefore both TRAF2 and cIAP1/2 each play

important roles in protecting cells from TNF induced death, and they must do so bound to each other.

TNF induced RIPK1 modification is dependent on a functional TRAF2-cIAP1/2 interaction but the TRAF2 RING domain is dispensable. Recent work has suggested that cIAP1/2 act as the E3 ubiquitin ligases to ubiquitylate RIPK1 following TNFR1 activation (14-16). However, it has also been proposed that the E3 ubiquitin ligase activity of TRAF2 mediates ubiquitylation of RIPK1 following TNFR1 signaling (28,29). Combined deletion of TRAF2 and TRAF5 (TRAF2/TRAF5 DKO) significantly prevented RIPK1 ubiquitylation in response to TNF stimulation and dramatically reduced cIAP1/2 binding to TNFR1 (Fig. 5). Thus the phenotype of the TRAF2/TRAF5 knock-out cells is consistent with both models and cannot distinguish between either. Restoration of WT TRAF2 expression in TRAF2/TRAF5 DKO MEFs restored RIPK1 ubiquitylation and cIAP1/2 recruitment to TNFR1, as expected (Fig. 5). However, TRAF2/5 DKO cells reconstituted with the TRAF2 CIM Δ 283-293 mutant did not recruit cIAP1 and did not restore RIPK1 ubiquitylation in response to TNF (Fig. 5). Reintroduction of TRAF2 Δ RING, which is still able to bind cIAPs (Fig.2) but lacks E3 ligase activity, also restored RIPK1 ubiquitylation and cIAP1/2 recruitment (Fig. 5). These data are consistent with the idea that cIAP1/2 are the most important E3 ligases for RIPK1, and fit the model whereby if TRAF2 does not recruit cIAP1/2 then RIPK1 fails to undergo TNF induced ubiquitylation and associates with FADD and caspase-8 to induce cell death. Intriguingly, although TNF induced RIPK1 ubiquitylation (Fig. 5), and NF- κ B function (see below) are retained upon loss of the TRAF2 RING domain, these cells are still susceptible to TNF killing (Fig. 4C).

The TRAF2 CIM, but not the TRAF2 RING domain, is required for efficient TNF induced NF- κ B activation. As previously described (24,53), TNF induced NF- κ B activation in TRAF2 knockout cells was similar to wildtype cells, as determined by NF- κ B reporter assays and Western blotting for p65 phosphorylation and I κ B α (Fig. 3A, 6A). Interestingly, we consistently observed that cIAP1^{-/-}, TRAF2^{-/-}, and TRAF2/TRAF5 DKO MEFs that have been immortalised with the

large SV40T antigen have increased levels of basal phosphorylated p65 when compared to WT cells (Fig. 6A, 6B) (17,19). However, this does not correlate with increased basal nuclear p65 levels in any of these cell lines (Fig. 6C, 6D, 7D) (17).

TRAF2 and TRAF5 are redundant with respect to TNF induced activation of NF- κ B, because deletion of both genes is required to prevent it (54). To examine the role of the TRAF2-cIAP1/2 interaction in TNF induction of NF- κ B we therefore complemented TRAF2/TRAF5 double knockout (DKO) MEFs with TRAF2 WT, CIM mutant, and RING domain deletion constructs. TRAF2/TRAF5 DKO cells were significantly impaired in TNF induced p65 phosphorylation, I κ B α degradation and translocation of p65 to the nucleus (Fig. 6B, 6C), and complementation with WT TRAF2 restored all these NF- κ B associated functions (Fig. 6B, 6C). While the TRAF2 CIM mutant EVE292AVA, that retains weak cIAP1/2 binding (Fig. 2B, 2D), partially restored TNF induced NF- κ B function (Fig. 6B, 6C) the TRAF2 Δ 283-293 CIM mutant was unable to restore either I κ B α degradation or nuclear translocation of p65 in response to TNF (Fig. 6B, 6C).

It has recently been suggested that TNF induced K63-linked ubiquitylation of TRAF2 on lysine 31 regulates binding of TAB2/TAB3 to TRAF2 and is required for proper TNF induced IKK and NF- κ B activity (55). If this model is correct then complementation of TRAF2/TRAF5 DKO cells with a Δ RING TRAF2 mutant, which lacks residues 1-87, should fail to restore TNF induced NF- κ B function. However, we observed that TRAF2/TRAF5 DKO MEFs complemented with Δ RING TRAF2 restored TNF induced p65 phosphorylation, I κ B degradation and translocation of p65 to the nucleus to a similar level as observed when TRAF2/TRAF5 DKO MEFs were complemented with WT TRAF2 (Fig. 6B, 6C).

Although rapid TNF induced nuclear translocation of p65 is mostly lost in TRAF2/TRAF5 DKO MEFs when compared to WT cells, some p65 nuclear localization could be detected after 60 minutes of TNF stimulation (Fig. 6D). Because immunoprecipitated TNFR1 from TRAF2/ TRAF5 DKO cells still bound low levels of cIAP1/2 (Fig. 5), we speculated that the late nuclear translocation of p65 in

TRAF2/TRAF5 DKO cells observed by immunofluorescence could be caused cIAP1/2 function. Consistent with this idea, when we depleted cIAP1/2 from TRAF2/TRAF5 DKO cells using the IAP antagonist Compound A (19), p65 translocation to the nucleus failed to occur, even 60 minutes after TNF stimulation (Fig. 6D).

Deletion of TRAF2 results in enhanced TNF induced c-FLIP degradation. Previous work has demonstrated that in the absence of NF- κ B signaling in TRAF2/TRAF5 DKO or p65 knockout cells the lack of TNF induced c-FLIP_L expression sensitises to TNFR1 killing (56). While it has previously been proposed that TRAF2 knockout cells are also sensitive to TNF due to reduced basal levels of c-FLIP_L (57) our Western blots suggested that both unstimulated TRAF2^{-/-} and TRAF2/TRAF5 DKO MEFs contain almost equivalent levels of c-FLIP_L when compared to WT cells (Fig. 7). However, when stimulated with TNF, both TRAF2^{-/-} and TRAF2/TRAF5 DKO cells displayed a dramatic loss of c-FLIP_L when compared to WT cells (Fig. 7). Hence despite the normal NF- κ B activation in TRAF2^{-/-} MEFs, c-FLIP_L is still efficiently depleted upon TNF stimulation and this probably accounts, at least partially, for their increased sensitivity to TNFR1 induced death.

DISCUSSION

Our study identifying and characterizing the role of the cIAP1/2 interacting motif of TRAF2 reveals that a key role of TRAF2 is in the recruitment of cIAP1/2 into signaling platforms. One of these is the NIK kinase complex that is essential for non-canonical NF- κ B activation. TRAF2 is a key molecule in the regulation of NIK stability as loss of TRAF2 results in NIK stabilisation, activation, and constitutive non-canonical NF- κ B signaling (17,52,58). Using TRAF2 CIM and RING mutants we complemented TRAF2 knockout cells and found that restoration of the normal low level of NIK and reduction of spontaneous NF- κ B activity required both the TRAF2 CIM and RING domain. These findings are consistent with recent reports showing that repression of non-canonical NF- κ B activity requires a complex of TRAF3, TRAF2 and cIAP1/2, with each component performing a distinct function (11,12,19,33). TRAF3 interacts with NIK and

also binds the TRAF2-cIAP1/2 module, thereby bringing cIAP1/2 into the proximity of NIK, resulting in cIAP1/2 mediated K48-linked ubiquitylation of NIK and its proteasomal degradation (11,12,33). These studies and our work here demonstrate that in addition to cIAP1/2, both the TRAF2 and TRAF3 RING domains are required for NIK degradation.

Our data also shows that TNF induced nuclear translocation of the canonical NF- κ B subunit p65 is significantly delayed in TRAF2/TRAF5 DKO cells, which agrees with previously reported NF- κ B electrophoretic mobility gel shift assays and *in vitro* IKK activity assays performed on TRAF2/TRAF5 DKO cells (54,55). cIAP1/2 have also been implicated in canonical NF- κ B signaling following TNFR1 activation, and both cIAP1/2 and TRAF2 have been suggested to be the E3 ligases required for ubiquitylation of RIPK1 (10,14-16,28,29). Ubiquitylated RIPK1 is believed to be the key signaling platform required for correct TNFR1 induced NF- κ B and a TRAF2 CIM mutant was unable to restore either RIPK1 ubiquitylation or normal NF- κ B signaling in response to TNF. Recently however we have shown that RIPK1 knockout MEFs are able to induce NF- κ B in response to TNF (L.W. and J.S, submitted). We therefore believe that RIPK1 ubiquitylation serves as a marker for TNF induced activation of NF- κ B but is not a requirement. While our work here and recent studies therefore suggest a role for cIAP1/2 in normal canonical NF- κ B responses initiated by TNF stimulation (10,15,16), it remains unclear what their key targets might be given that RIPK1 itself is dispensable for TNF induced NF- κ B.

The ubiquitylation of RIPK1 by cIAP1/2 has been shown to prevent it associating with FADD and caspase-8, thereby limiting TNFR1 induced cell death (10,15,16). Consistent with this model, cIAP1 knockout MEFs have increased RIPK1 recruitment to TNFR1 and increased sensitivity to TNF killing compared to wild type MEFs (10,19). Similarly, IAP antagonist treatment that depletes cIAP1/2 causes a loss of RIPK1 modification following TNF stimulation (10,16). Following TNFR1 activation we observed increased amounts of RIPK1 bound to TNFR1 and decreased RIPK1 ubiquitylation in TRAF2/TRAF5 DKO cells. TRAF2/TRAF5 DKO cells reconstituted with either WT TRAF2 or a TRAF2 Δ RING mutant,

but not a TRAF2 CIM mutant, regained RIPK1 ubiquitylation. These results show that TRAF2 dependent TNF-induced RIPK1 ubiquitylation requires an intact TRAF2-cIAP1/2 interaction. Combined with the fact that the TRAF2 RING domain was dispensable for RIPK1 ubiquitylation, our data support the model that cIAP1/2 are the critical E3 ligases required for RIPK1 ubiquitylation.

While we show that TRAF2 must recruit cIAP1/2 in order to properly activate NF- κ B, TRAF2 is not just a passive adaptor, because TRAF2's RING domain is required for imparting resistance to induction of apoptosis by TNF. Our results with TRAF2^{-/-} cells and those reconstituted with a TRAF2 Δ RING mutant, demonstrates an apparently normal NF- κ B induction combined with sensitivity to TNF killing. While decreased cIAP1/2 recruitment into the TNFR1 signalling complex in TRAF2^{-/-} MEFs may lead to less RIPK1 ubiquitylation and theoretically promote TNF induced cell death, our data shows that TRAF2 Δ RING restores cIAP1/2 binding to TNFR1 and RIPK1 ubiquitylation, yet fails to restore TNF resistance. The almost normal canonical NF- κ B response on the background of increased non-canonical NF- κ B signalling in TRAF2^{-/-} MEFs ((17) and this work) may render them sensitive

to TNF but this remains to be tested. Alternatively, an enhancement in c-FLIP_L degradation may render TRAF2^{-/-} cells more susceptible to TNFR1 induced death. To this end, we observed a dramatic decrease in c-FLIP_L levels in TRAF2^{-/-} cells upon TNF stimulation which is likely to contribute to their enhanced TNF sensitivity. While this data fits current models where active caspase-8 has been shown efficiently cleave c-FLIP_L (59), it remains to be determined how c-FLIP_L levels are regulated by the presence of TRAF2.

Our results here define a TRAF2-cIAP1/2 interacting motif and provide independent support for recent studies with IAP antagonist compounds that demonstrate a fundamental role for cIAP1/2 in regulating TNF signaling. Reconstitution of TRAF2 and TRAF2/TRAF5 DKO cells with a TRAF2 Δ RING mutant has however raised new questions. This mutant was able to recapitulate wild type TRAF2's ability to activate canonical NF- κ B in response to TNF in TRAF2/TRAF5 DKO cells but was unable to protect TRAF2 knock-out cells from the cytotoxic activity of TNF. It will therefore be interesting to determine what the additional cytoprotective function of TRAF2 is.

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FOOTNOTES

We would like to thank Hiroyasu Nakano for TRAF2/TRAF5 double knock-out MEFs, David Baltimore for pFU and lentiviral packaging constructs, Jurg Tschopp for generous gifts of materials and Robert Brink (Garvan Institute) for critically reading the manuscript.

JS is supported by NHMRC grants #433013, 541901 and 541902. DV is an Australian Fellow, funded by the Leukemia and Lymphoma Society and NHMRC grant #461221. PDM is a recipient of a Health Sciences Career Development Award (University of Otago). EFL was funded by The Cancer Council Victoria. JEV is supported by a Human Frontiers Science Program Fellowship.

The abbreviations used are: cIAP, cellular inhibitor of apoptosis; IAP, inhibitor of apoptosis; NF- κ B, nuclear factor-kappa-light-chain-enhancer of activated B cells; TNF, tumor necrosis factor; TNFR1, tumor necrosis factor receptor-1; TRAF, TNF receptor associated factor; RIPK1, receptor interacting protein-1; TNFSF, TNF superfamily; I κ B, inhibitor NF- κ B; IKK, I κ B kinase; RING, really interesting new gene; TAK1, TGF- β -activated kinase-1; TAB, TAK1 binding protein; NEMO, NF- κ B essential modulator.

FIGURE LEGENDS

Figure 1. Identification of a TRAF2-cIAP1/2 Interacting Motif (CIM). *A*, Schematic of TRAF2 domain structure and localization of the CIM. *B*, and *C*, The TRAF-N domain of TRAF2 interacts with the BIR1 domain of cIAP1. 293T cells were co-transfected with the indicated HA-cIAP1 constructs and FLAG-TRAF2 (*B*) or FLAG-TRAF2-N (*C*) and their interaction examined by FLAG-immunoprecipitation and Western blot. M22 and M39 refer to BIR1 deletion constructs lacking the first 22 and 39 residues respectively. *D*, Conservation within the TRAF2-N domain. The TRAF-N domain of TRAF2 was aligned from the indicated organisms (accession numbers in brackets) and compared with the same region of human TRAF1 (bottom line). Identical residues are shaded in black and conserved residues in grey. Residues mutated in TRAF2 to examine the effect on cIAP1/2 binding are highlighted with an asterisk and the deletion (283-293) and truncation mutants (D310-stop) also indicated.

Figure 2. TRAF2 residues 292EVE294 and 283-293 are important for cIAP1 and cIAP2 binding to TRAF2. *A*, Determination of TRAF2 residues required for cIAP1 binding. 293T cells were transfected with the indicated FLAG-TRAF2 constructs for 48 hrs and then immunoprecipitated from cell lysates. Binding of endogenous cIAP1 to FLAG-TRAF2 was determined by Western blot. The asterisk indicates IgG light chain cross reactivity. *B*, Mutation of both TRAF2 E292 and E294 is required to reduce cIAP1 and cIAP2 binding. 293T cells were transfected with the indicated FLAG-TRAF2 constructs in addition to cIAP2. FLAG-TRAF2 complexes were immunoprecipitated and TRAF2 binding to endogenous cIAP1 and ectopically expressed cIAP2 determined by Western blot. Asterisk indicates a non-specific band. *C*, FLAG-TRAF2 CIM mutants showing reduced cIAP1/2 binding interact with RIPK1 and TRADD. 293T cells were transfected with FLAG-TRAF2 constructs and immunoprecipitated as in (*A*) and binding to RIPK1 and TRADD determined by Western blot. *D*, The TRAF2-N domain binds directly to the cIAP1 BIR1 domain. The indicated GST proteins were incubated with purified WT or mutant TRAF2-N domain protein as indicated and, following GST immunoprecipitation, binding of the TRAF2-N domain (T2N) detected by Coomassie staining.

Figure 3. The TRAF2 CIM region 283-293 and RING domain are important for repression of constitutive non-canonical NF- κ B activity caused by TRAF2 deletion. *A*, TRAF2 deletion results in constitutive NF- κ B activity that is further enhanced by TNF stimulation. TRAF2 was deleted from parental TRAF2^{LoxP/LoxP} conditional knockout MEFs containing a lentiviral NF- κ B GFP reporter by infection with a lentivirus harboring a Cre recombinase expressing plasmid. TRAF2 parental WT and knockout NF- κ B GFP reporter cells were stimulated with TNF for 24 hrs and reporter activity measured by flow cytometry. Blue colouring: WT unstimulated cells. Red colouring: TRAF2^{-/-} unstimulated cells. Green colouring: TNF stimulated cells. *B*, WT but not CIM mutated TRAF2

restores basal NF- κ B GFP reporter activity in TRAF2^{-/-} cells. TRAF2 expression in the TRAF2 conditional knockout MEFs described in (A) was restored with the indicated WT and mutant constructs. Basal NF- κ B reporter activity was measured by flow cytometry 72 hrs post-restoration of TRAF2 expression. Yellow colouring: Parental WT MEFs. Light blue colouring: TRAF2^{-/-} MEFs. Red colouring: Uninduced TRAF2^{-/-} cells reconstituted with the indicated inducible constructs. Blue colouring: Induced TRAF2^{-/-} cells reconstituted with the indicated inducible constructs. The experiments shown are representative of results obtained on three independent occasions. C, TRAF2 deletion results in constitutive non-canonical NF- κ B activity that is not reduced to normal levels by expression of TRAF2 CIM Δ 283-293 or RING domain mutants. TRAF2^{-/-} cells described in (A) were infected with the indicated inducible lentiviral TRAF2 constructs and TRAF2 expression induced in independent clones. 72 hrs post-induction, cells were harvested and analyzed by Western blot for the indicated proteins.

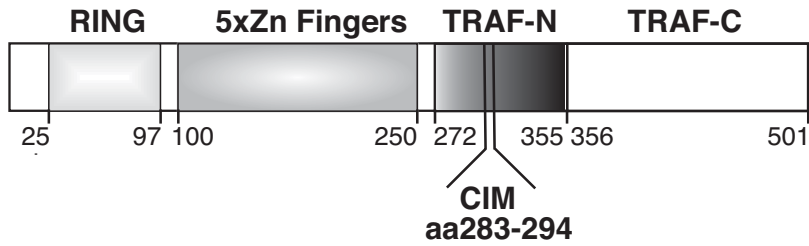
Figure 4. TRAF2 CIM residues 292EVE294 and 283-293 are required for TRAF2 mediated protection against TNF induced death. A, Immortalized TRAF2^{-/-} cells are sensitized to TNF but not FasL or TRAIL induced apoptosis. SV40 large T immortalized TRAF2 conditional knockout MEFs were infected with Cre recombinase to delete TRAF2. Parental TRAF2^{LoxP/LoxP} and TRAF2^{-/-} MEFs were treated with TNF (60 ng/ml), FasL (10 ng/ml), TRAIL (1 μ g/ml) or Compound A (500 nM) as indicated for 24 hrs and cells death quantified by PI staining and flow cytometry. Error bars are SEM of at least three independent experiments. B, Restoration of TRAF2 expression in TRAF2^{-/-} cells restores TNF resistance. TRAF2^{-/-} MEFs were infected with a lentiviral construct containing 4HT inducible WT murine TRAF2. TRAF2 expression was induced by the indicated doses of 4HT (Western blot, bottom) and cell resistance to TNF (60 ng/ml) killing after 24 hrs assessed by PI staining and flow cytometry. C, TRAF2 292EVE294 and Δ 283-293 mutants do not protect against TNF killing in TRAF2^{-/-} MEFs. TRAF2^{-/-} cells were infected with the indicated 4HT inducible lentiviral TRAF2 constructs and TRAF2 expression induced for 48 hrs (see Fig. 3C for TRAF2 expression levels). Cells were then treated with TNF (60 ng/ml) for a further 24 hrs and cell death compared to their uninduced counterparts by PI staining and flow cytometry. Error bars are SEM of 3-5 independent experiments for several different clones.

Figure 5. The TRAF2 CIM is important for TNF induced RIPK1 ubiquitylation but the TRAF2 RING domain is dispensable. The indicated MEFs were stimulated with FLAG-TNF (1 μ g/ml) for 5 minutes and the TNFR1 signaling complex precipitated with anti-FLAG antibody as described in the *Experimental Procedures*. The eluted TNFR1 protein complex was examined by Western blot for the indicated proteins. To precipitate unstimulated TNFR1 (time=0) FLAG-TNF was added to cell lysates.

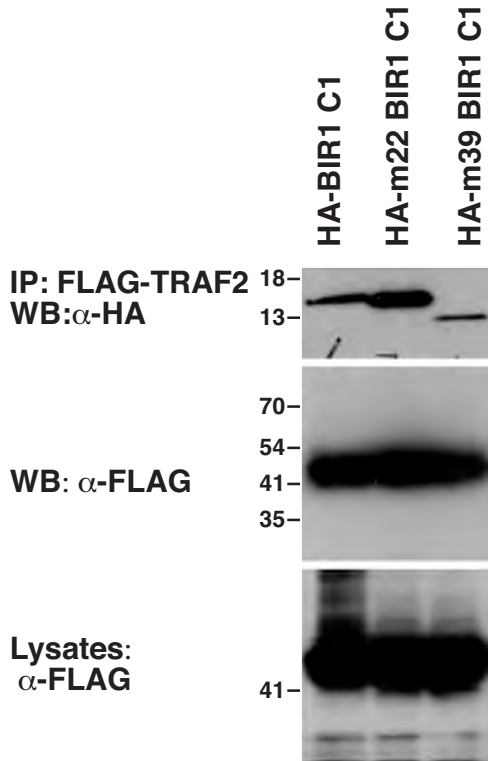
Figure 6. The TRAF2 CIM is important for efficient TNF induced NF- κ B activation but the TRAF2 RING domain is dispensable. A, TNF induction of NF- κ B in TRAF2 knockout MEFs. WT and TRAF2^{-/-} cells were treated with TNF (60 ng/ml) for the indicated times and cell lysates analyzed by Western blot. B, Restoration of the normal NF- κ B response to TNF in TRAF2/TRAF5 double knockout MEFs requires the TRAF2 CIM but not the TRAF2 RING domain. The indicated cell types were treated with TNF (60 ng/ml) for the time periods shown and cell lysates analyzed by Western blot. C, TNF induced translocation of p65 into the nucleus requires the TRAF2 CIM but not the TRAF2 RING domain. The indicated MEF cell lines were stimulated with TNF (60 ng/ml) for 20 min and p65 localization analyzed by immunostaining and fluorescence microscopy. DKO: TRAF2/TRAF5 double knockout. D, Delayed p65 nuclear translocation in TRAF2/TRAF5 DKO MEFs is prevented by IAP antagonist depletion of cIAP1/2. WT and TRAF2/TRAF5 DKO MEFs were treated with 60ng/mL of TNF for the indicated times with or without the IAP antagonist, compound A, and p65 localisation examined by immunofluorescence microscopy.

Figure 7. Deletion of TRAF2 increases c-FLIP_L loss upon TNF stimulation. The indicated MEF cell lines were stimulated with TNF for 0, 1 or 2 hours and cell lysates examined by Western blot for the proteins shown.

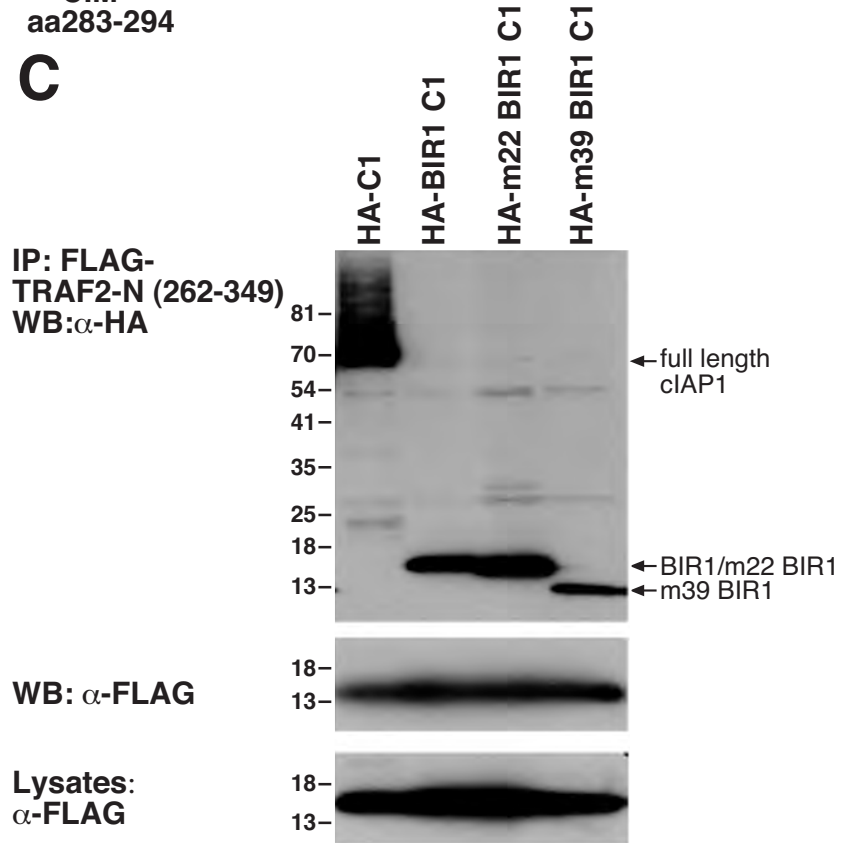
A



B



C



D

Goldfish (CAC92653)
Trout (NP_001117865)
Human (NP_0066961.2)
Monkey (XP_001092078)
Mouse (NP_033448)
Rat (NP_001101285)
Dog (XP_857289)
Horse (XP_001497958)
Human Traf1 (NP_005649.1)



Figure 1

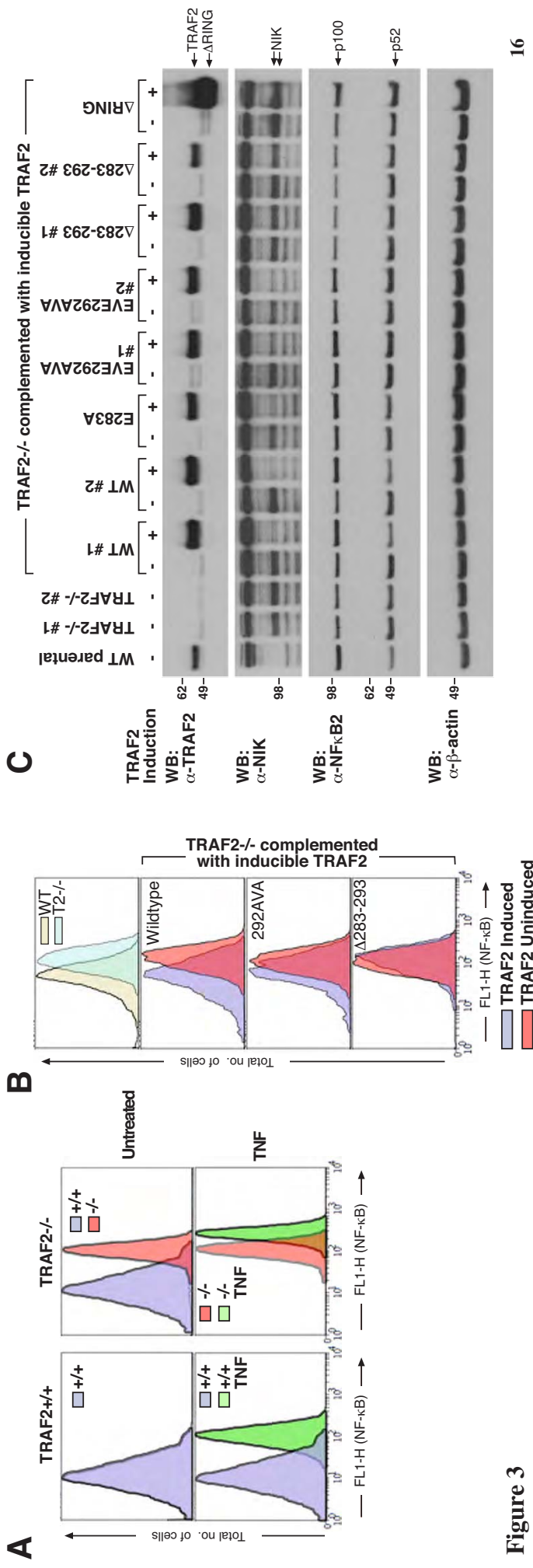


Figure 3

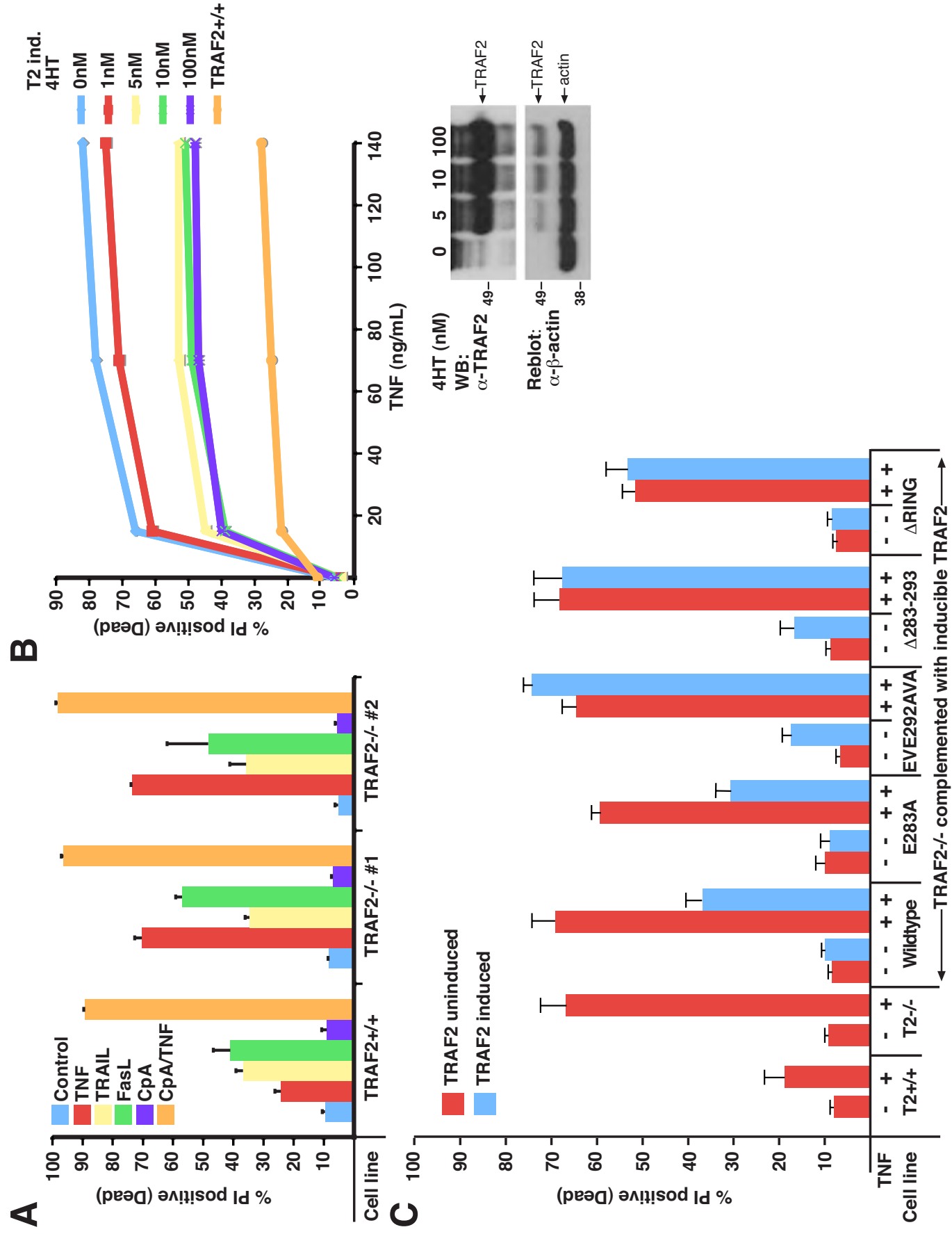


Figure 4

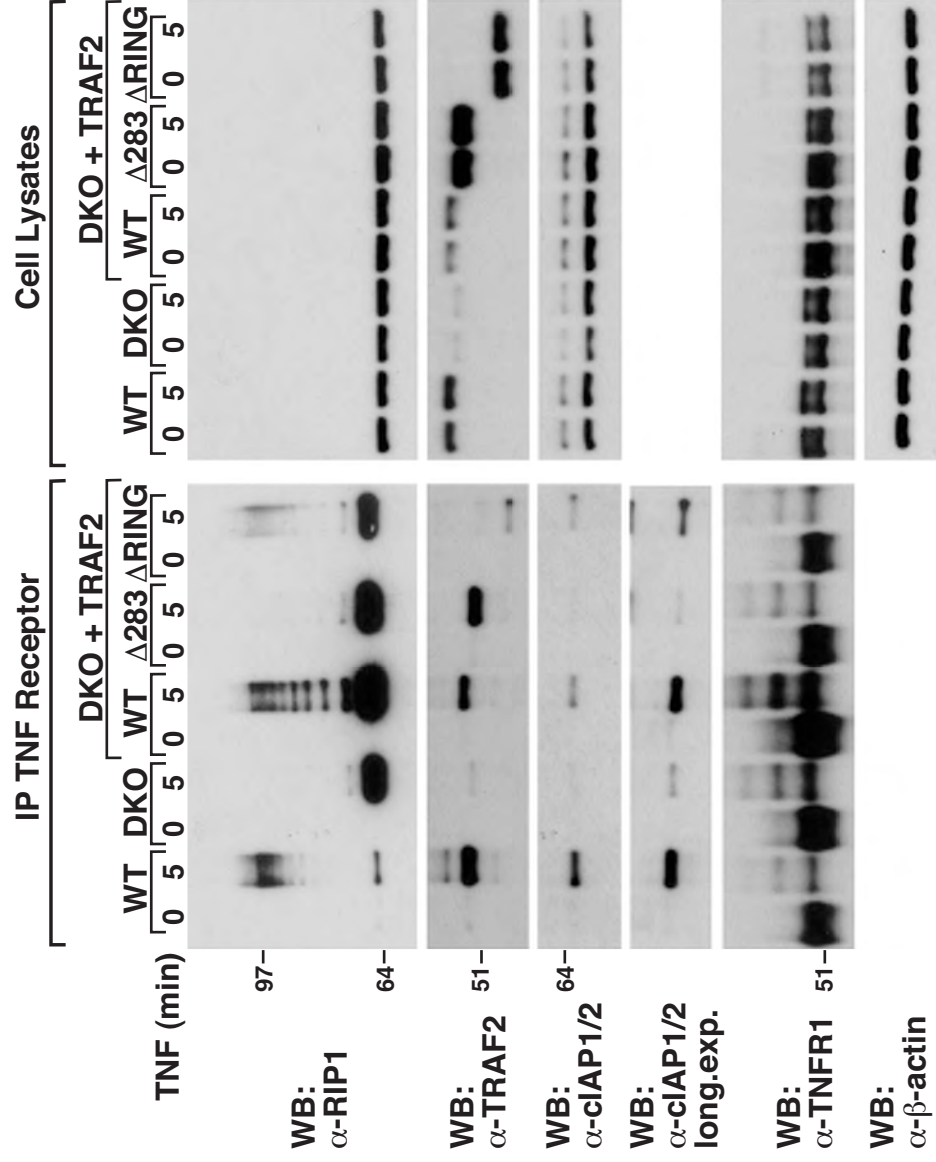


Figure 5

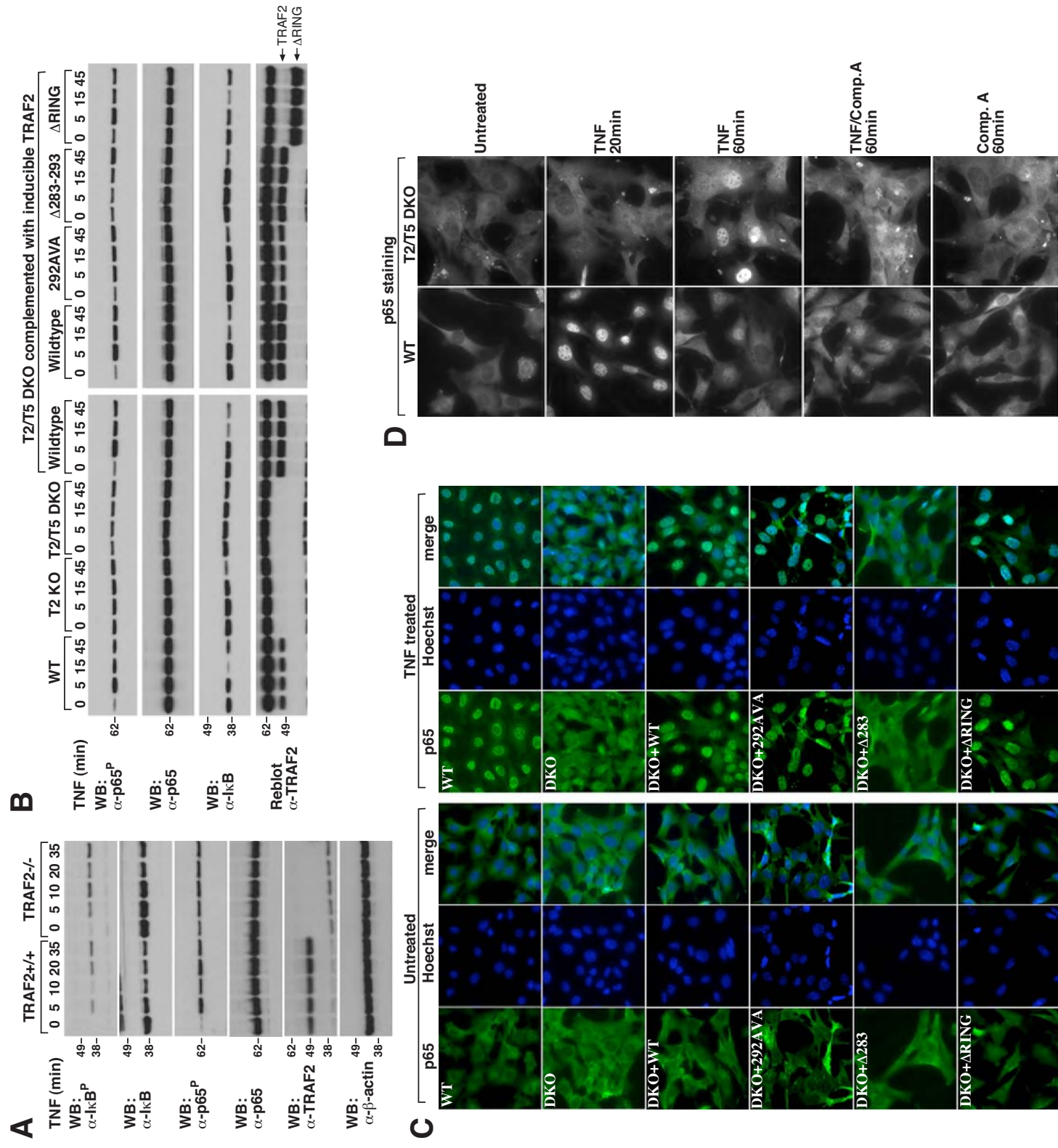


Figure 6

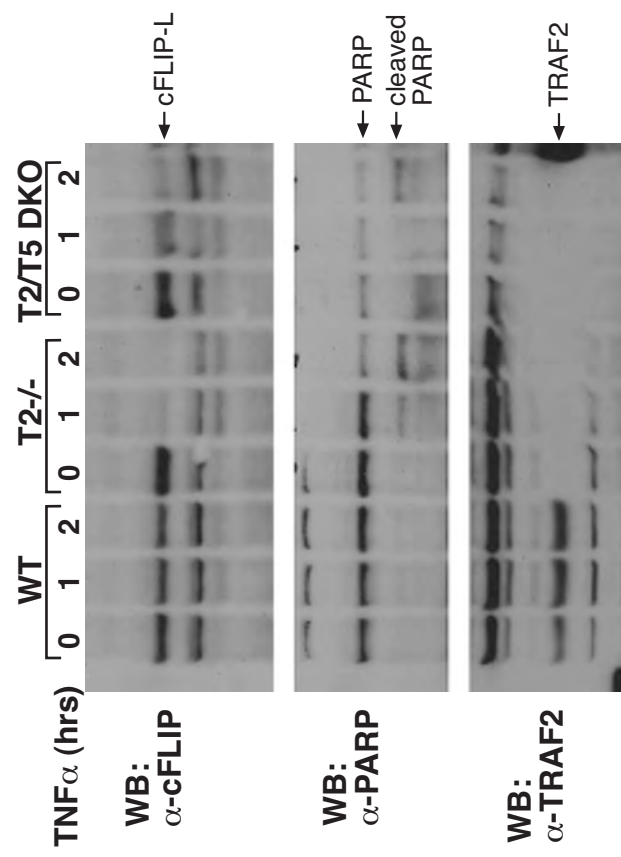


Figure 7